

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : C12Q 1/68, G01N 33/576 // C07H 21/04, C07K 7/00	A1	(11) International Publication Number: WO 94/26932 (43) International Publication Date: 24 November 1994 (24.11.94)
(21) International Application Number: PCT/US94/05407 (22) International Filing Date: 13 May 1994 (13.05.94) (30) Priority Data: 08/061,694 13 May 1993 (13.05.93) US (71) Applicant: UNITED STATES OF AMERICA, as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES National Institutes of Health [US/US]; Office of Technology Transfer, Box OTT, Bethesda, MD 20892 (US). (72) Inventors: FIELDS, Howard, A.; 1823 Jackson Creek Drive, Marietta, GA 30068 (US). KHUDYAKOV, Yury, E.; 1402-F Druid Valley Drive, Atlanta, GA 30239 (US). (74) Agents: NEEDLE, William, H. et al.; Needle & Rosenberg, Suite 1200, 127 Peachtree Street N.E., Atlanta, GA 30303-1811 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: NUCLEIC ACID TAGGED IMMUNOASSAY (57) Abstract Provided is a method for detecting the presence of an antigen in a specimen from a subject comprising the steps of (a) binding antigens in a specimen from the subject to a solid support; (b) contacting the bound antigens with a specifically immunoreactive ligand, wherein the ligand is linked to an oligonucleotide, having a known sequence, under conditions that permit reaction of the oligonucleotide-linked ligand with the bound antigen; (c) removing unreacted oligonucleotide-linked ligand; and (d) detecting the presence of the oligonucleotide, the presence of the oligonucleotide indicating the presence of the antigen in the subject. A method for detecting the presence of an antibody in a specimen from a subject is also provided. A composition comprising an immunoreactive polypeptide linked to an oligonucleotide is also provided.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

NUCLEIC ACID TAGGED IMMUNOASSAY

BACKGROUND OF THE INVENTION

5 The detection of nucleic acids is utilized for the diagnosis of many diseases. Efficient cost effective detection is critical for the diagnosis of viruses and other microorganisms. Techniques such as the polymerase chain reaction (PCR) have revolutionized effective
10 detection of microorganisms. Despite these advances, there still remain many problems with the sensitivity of these methods.

 The coupling of the technology of PCR with
15 antigen detection systems to achieve greater sensitivity of detection was disclosed in Sano et al., "Immuno-PCR: Very Sensitive Antigen Detection by Means of Specific Antibody-DNA Conjugates," Science 258:120-122 (October 1992). Sano describes a method, whereby a known antibody
20 is allowed to react with a sample (e.g., an antigen bound to a solid phase), unbound antibody is removed, then a biotinylated double stranded linear marker DNA molecule is attached to any resulting solid-phase bound antigen-IgG antibody complexes by means of a linker molecule having
25 affinity for both biotin and the Fc of IgG molecules, resulting in the formation of an antigen-antibody-DNA conjugate. This attached DNA can then be amplified by PCR with appropriate primers, and such amplified DNA is detected by agarose gel electrophoresis, resulting in
30 enhanced sensitivity of the detection of the antigen. The methods disclosed Sano by have the disadvantage that they cannot be used to detect antibodies from a sample, since the antibody must be selectively bound to a solid support.

35 Recently, another disclosure entitled "Immuno-PCR with a commercially available avidin system" (Ruzicka, V., Marz, W., Russ, A., Gross, W., Science 260: 689-699, April

30, 1993), described the use of a streptavidin-biotin linkage to put together an antibody and a PCR fragment. The method of Ruzicka et al. has the disadvantage of using oligonucleotides derived from organismal DNA.

5

The present invention provides the advantages of increased sensitivity and in addition overcomes the above disadvantages by providing an improved nucleic acid tagged immunoassay for the detection of antigens and antibodies in specimens.

10

SUMMARY OF THE INVENTION

The present invention provides a method for detecting the presence of an antigen in a specimen from a subject comprising the steps of (a) binding antigens in a specimen from the subject to a solid support; (b) contacting the bound antigens with a specifically immunoreactive ligand, wherein the ligand is linked to an oligonucleotide, having a known sequence, under conditions that permit reaction of the oligonucleotide-linked ligand with the bound antigen; (c) removing unreacted oligonucleotide-linked ligand; and (d) detecting the presence of the oligonucleotide, the presence of the oligonucleotide indicating the presence of the antigen in the subject.

A method for detecting the presence of an antibody in a specimen from a subject is also provided. The method can include the following steps: (a) binding antibodies in a specimen from the subject to a solid support; (b) contacting the bound antibodies with a specifically immunoreactive antigen, wherein the antigen is linked to an oligonucleotide, having a known sequence, under conditions that permit reaction of the oligonucleotide-linked antibody with the bound antibody from the subject; (c) removing unreacted oligonucleotide-linked antigen; and (d) detecting the presence of the oligonucleotide, the

30

35

presence of the oligonucleotide indicating the presence of the antibody in the subject.

The invention also includes a composition comprising
5 an oligonucleotide of known sequence linked to a
specifically immunoreactive antigen or ligand. The
ligand-oligonucleotide compositions and antigen-
oligonucleotide compositions of the invention are each
referred to herein as an initiator of nucleic acid
10 amplification (INAA).

DETAILED DESCRIPTION OF THE INVENTION

Nucleic acid tagged immunoassay (NATIA) is a
15 technique for the detection of an antigen-antibody
interaction using methods for the amplification of nucleic
acids. This method can be used for the detection of very
diluted antigens or antibodies because of the high
efficiency and sensitivity of the nucleic acid
20 amplification step. Sensitivity of NATIA can be 1,000 -
1,000,000 times greater than conventional enzyme
immunoassays or radioimmunoassays.

NATIA is a method for detecting the presence of an
25 antigen in a specimen from a subject comprising the steps
of (a) binding antigens in a specimen from the subject to
a solid support; (b) contacting the bound antigens with a
specifically immunoreactive ligand, wherein the ligand is
linked to an oligonucleotide, having a known sequence,
30 under conditions that permit reaction of the
oligonucleotide-linked ligand with the bound antigen; (c)
removing unreacted oligonucleotide-linked ligand; and (d)
detecting the presence of the oligonucleotide, the
presence of the oligonucleotide indicating the presence of
35 the antigen in the subject.

The term "immunoreactive" means capable of binding or otherwise associating nonrandomly with an antigen or antibody. "Specifically immunoreactive" as used herein describes an antibody or other ligand or an antigen that
5 does not cross react substantially with any antigen or antibody other than the one specified. The term "ligand," as used herein, includes, for example, antibodies (monoclonal or polyclonal), immunoreactive fragments of such antibodies and other molecules capable of binding or
10 otherwise associating nonrandomly with an antigen or antibody. "Antigen," as used herein refers to immunoreactive proteins and immunoreactive polypeptide fragments thereof, and includes disease markers.

15 A method for detecting the presence of an antibody in a specimen from a subject is also provided. The method can include the following steps: (a) binding antibodies in a specimen from the subject to a solid support; (b) contacting the bound antibodies with a specifically
20 immunoreactive antigen, wherein the antigen is linked to an oligonucleotide, having a known sequence, under conditions that permit reaction of the oligonucleotide-linked antibody with the bound antibody from the subject; (c) removing unreacted oligonucleotide-linked antigen; and
25 (d) detecting the presence of the oligonucleotide, the presence of the oligonucleotide indicating the presence of the antibody in the subject.

In the methods of the invention the step of binding
30 antigens or antibodies in a specimen to a solid support can comprise any of the known methods for attaching an antigen to a solid surface. Examples of such methods are provided in the Examples. The solid support used in the methods can, for example, include microtiter plates or
35 other labware, beads, magnetic particles or other solid material. The specimen can comprise any body fluid which would contain the antigen, a cell containing the antigen

or the antibody, such as blood, plasma, serum, saliva and urine sputum, mucus, gastric juice and the like. The specimen can also be stool or tissue from the subject.

5 In the method for detecting an antigen in a specimen from the subject, the ligand can be linked to an oligonucleotide by biotin-streptavidin-biotin as known in the art and described below in the Examples. In the method for detecting antibody in a specimen from the
10 subject, the oligonucleotide and the antigen can be linked covalently by chemical reaction or non-covalently linked as by biotin-streptavidin. Methods for generating the covalent linkage of the oligonucleotide to the antigen are well known in the art and exemplified in the Examples.

15 In the method of detecting antigen, the antigens bound to the solid support are contacted with a specifically immunoreactive ligand linked to an oligonucleotide as detailed below in the Examples. In the method for detecting the presence of an antibody in a
20 specimen from a subject the bound antibodies are contacted with a specifically immunoreactive antigen, wherein the antigen is linked to an oligonucleotide. The conditions of the contacting step that permit reaction of the oligonucleotide-linked ligand with the bound antigen are
25 exemplified below. The oligonucleotide used in the method has a known sequence, which permits detection of the presence of the oligonucleotide linked to the specifically immunoreactive ligand or antigen. The unreacted oligonucleotide-linked ligand is removed by washing the
30 solid support to which the antigen is attached.

In the step of detecting the presence of the oligonucleotide having a known sequence, the oligonucleotide is amplified prior to detection. The
35 amplification can be by polymerase chain reaction, ligase chain reaction or other method for amplifying nucleic acids. Other amplification methods that can be used in

the present methods are well known in the art and include 3SR amplification (Guatelli et al. "Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication" Pros. Natl. Acad. Sci. USA 87:1874-1878, 1990); amplification with Q-beta replicase (Wolcott, M. J. "Advances in nucleic acid-based detection methods", Clinical Microbiology Reviews, 5(4):370-386, 1992); and strand displacement amplification (Walker et al. "Isothermal in vitro amplification of DNA by restriction enzyme DNA polymerase system" Proc. Natl. Acad. Sci. USA 89:392-396, 1992). The oligonucleotide can then be detected by detecting the product of amplification.

15 The methods that can be used to detect the amplified product will depend on the amplification method generally and the primers used. For example, in an amplification method using a primer labeled with a detectable moiety, the presence of the detectable moiety indicates the
20 presence of amplification product. The detectable moiety can be directly detectable (e.g., a radioactive or fluorescent marker) or indirectly detectable, such as by an enzymatic reaction producing a detectable product (e.g., horseradish peroxidase) as described in the
25 Examples.

Other methods of detecting the presence of the oligonucleotide include the detection of RNA transcripts generated from the oligonucleotide using RNA polymerase.
30 The transcript can then be detected using standard methods, such as by gel electrophoresis followed by northern blotting or by the methods used to detect DNA, such as those described in the Examples. Because the oligonucleotide is linked to a specifically immunoreactive
35 ligand or antigen, the presence of the oligonucleotide indicates the presence in the subject of the respective antigen or ligand specifically reactive therewith.

A method for detecting current or previous hepatitis C virus (HCV) infection in a subject is provided. Specifically the method can comprise the steps of binding antibodies in a specimen from the subject to a solid support and contacting the bound antibodies with an oligonucleotide covalently linked to the polypeptide consisting essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:1 under conditions that permit the reaction of that composition with an antibody from the subject. "Consisting essentially of" can include minor amino acid substitutions, insertions and deletions which do not substantially diminish the polypeptides antigenicity. After removing the unreacted oligonucleotide-linked polypeptide, the presence of the oligonucleotide is detected, the presence of the oligonucleotide indicates the reaction of the polypeptide and an antibody from the subject. The reaction indicates current or previous hepatitis C virus infection. The binding, contacting, removing and detecting steps in the NATIA for detecting HCV infection are carried out as described above and in the Examples.

Because the invention provides methods and compositions capable of detecting very small quantities of antigen, antibody or other marker of disease, the invention also provides a method for the early detection of malignancies in a manner according to the methods described above and in the Examples with the antigen or ligand selected based on the disease to be detected. Existing methods do not allow for the routine detection of cancer tumors at the stage when it may be easily cured. NATIA is a very sensitive method for the detection of antigen-antibody binding. This method has wide applications in the development of diagnostics for many diseases, especially those with low levels of circulating markers of infection. Increased sensitivity of the method improves the specificity of many immunodiagnostic

reactions by allowing these tests to be run at very high specimen dilutions, which result in decreased detection of non-specific reactions. The increased sensitivity will compensate for any signal lost as the result of diluting
5 the test specimen.

The invention also includes a composition comprising an oligonucleotide of known sequence linked to a specifically immunoreactive antigen or ligand. The
10 ligand-oligonucleotide compositions and antigen-oligonucleotide compositions of the invention are each referred to herein as an initiator of nucleic acid amplification (INAA). Particularly, an example of the composition comprises an oligonucleotide covalently linked
15 to the polypeptide consisting essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:1. This polypeptide is a specifically immunoreactive antigen of hepatitis C virus (HCV) and can be used in the method of detecting current or previous HCV infection.

20
Another example of a composition of the present invention comprises an oligonucleotide covalently linked to the polypeptide consisting essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID
25 NO:2. This polypeptide is a specifically immunoreactive antigen of hepatitis E virus (HEV) and can be used in a method of detecting current or previous HEV infection, comprising the same general steps as described above for HCV.

30
An isolated nucleic acid comprising essentially the nucleotide sequence defined in the Sequence Listing by SEQ ID NO:3 is also provided. The nucleic acid can be used as the oligonucleotide in the NATIA methods of the present
35 invention. Thus the nucleic acid can be linked to a number of antigens or antibodies to form the INAA.

For example, a composition of the invention can comprise an immunoreactive polypeptide covalently linked to the above nucleic acid. More specifically, the immunoreactive polypeptide of the composition can consist essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:1. Alternatively, the polypeptide can consist essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:2.

10 The main advantage of the NATIA is the extremely efficient method of enhancing a signal initiated by an antigen-antibody binding. Another advantage is a shift from protein-protein interactions (antigen-antibody recognition) to nucleic acid synthesis. This shift
15 significantly diminishes the "noise" often associated with antigen-antibody interactions. Non-specific protein-protein interaction will not be detected because of the use of nucleic acid amplification. In addition to synthetic peptides, any protein such as viral or bacterial
20 antigens or antigenic fragments of antigens, or antibodies, can be conjugated to a nucleic acid tag.

 The following advantages of the use of the invention derive from the use of synthetic oligonucleotides for
25 amplification instead of natural DNA fragments:
(1) short synthetic oligonucleotides result in better efficiency of amplification; (2) the structure of the oligonucleotide can be modified in any way to facilitate amplification (weak secondary structure) or detection,
30 particularly for the solid-phase detection of amplification products; (3) simplicity of introducing specific chemical modifications for chemical attachment of oligonucleotides to immunoreactive components of NATIA;
(4) greater ability to be specifically designed for the
35 use in amplification reactions other than PCR. Lack of secondary structure (if desired) allows for the use of room temperature to initiate efficient isothermal

amplification reactions such as Q-beta polymerase specific amplification; and (5) with the use of synthetic peptides, the linkage of oligonucleotide and peptide can be chemical, the resulting composition can be easily purified and characterized and can be obtained in homogeneous form without significant contamination with unlinked oligonucleotide or antigen.

In addition to the advantage mentioned above the use of synthetic peptides for NATIA has a further advantage of the detection of an immunoreaction to one individual epitope, which is impossible with any other known antigen.

The following examples are intended to illustrate, but not limit, the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may be alternatively employed.

EXAMPLES

Detection of HCV and HEV Infection

Preparation of INAA.

As an example, a synthetic immunoreactive peptide of the sequence ANPPDHSAPLGVTRPSA (SEQ ID NO:1) containing an antigenically active region from the protein encoded by the open reading frame 3 (ORF3) of the hepatitis E virus (HEV), or a synthetic peptide of the sequence AFASRGNHVSPTHYVPESDA (SEQ ID NO:2) containing an antigenic epitope encoded by the hepatitis C virus (HCV), is covalently linked to a synthetic oligonucleotide. The sequence of the oligonucleotide may vary depending on the method of amplification chosen for detection. An example of oligonucleotide for use in the present invention has the following primary structure: 5'-
CCTCCCCTATCTTCTCCTCCCCAACCCCAAAAAAAAAAAAAAAAAA -3' (SEQ ID NO:3).

This oligonucleotide can be covalently attached by the 5'-terminus to the N- or C-terminal sequence of a synthetic peptide using well-known methods of conjugation. For example, using an amino-group attached to an

5 oligonucleotide during the synthesis of the oligonucleotide, this oligonucleotide may be covalently linked to a peptide by amino-(such as lysine, or N-terminal amino-group) or carboxy-group (such amino acids as glutamic or aspartic acid, or C-terminal group). There

10 are several routine protocols to carry out this conjugation. One of them is the use of the homobifunctional reagent disuccinimidyl suberate (Nucleic Acids Research 14(15):6115-6128, 1986, Jablonski et al. "Preparation of oligonucleotide-alkaline phosphate

15 conjugates and their use as hybridization probes"). This protocol allows for the coupling of the 5'-terminally located amino-group of an oligonucleotide to the amino-group at the N-terminus of a peptide. In another example, the 1,3-diisopropylcarbodiimide can be used in combination

20 with H-hydroxybenzotriazole for conjugation of the 5'-terminal amino group of an oligonucleotide to the C-terminal carboxy-group of a peptide. Other carbodiimide derivatives may be used to obtain this product. (see catalog of Pierce Company for additional examples of

25 suitable coupling agents). Other reactions well known for protein-protein coupling can also be used with routine modifications to link an oligonucleotide and a peptide. The covalent complex composed of the oligonucleotide and a

30 synthetic peptide is called an initiator of nucleic acid amplification (INAA).

Preparation of specimen.

Plastic tubes or microtiter wells are adsorbed with serum from a subject suspected of containing anti-HCV or

35 anti-HEV antibodies reactive with the synthetic HCV and HEV peptides, respectively. Briefly, a serum specimen was diluted 1:500 by phosphate buffered saline (PBS). 100 μ l

of the diluted specimen was applied to each microtiter plate well and incubated at room temperature overnight. Next day, the plates were washed thoroughly with PBS containing 1% bovine serum albumin, 0,05% Tween 20.

5

Immunoreaction of specimen and INAA.

The INAA is dissolved in 0.01 M PBS, pH 7.2, containing 1% bovine serum albumin, and 10% normal goat serum. Tubes or wells adsorbed with the specimen are
10 incubated with this solution for 30 min to 1 h to allow the synthetic peptides to specifically react with antibody in the specimen. After thorough washing of the tubes or wells to remove unbound INAA, the bound INAA containing an
15 available oligonucleotide having the specifically designed sequence remains in the tube or in the well attached to the solid support.

Amplification of the reacted oligonucleotide.

When the immunosorption reaction is carried out in
20 microtiter wells, the INAA must be dissociated from the surface by treatment with 0.2 M glycine, pH 2.5, for approximately 2 min, and transferred into plastic tubes for amplification. The oligonucleotide can be used for the initiation of the amplification by several different
25 methods, such as the polymerase chain reaction (PCR) or ligase chain reaction (LCR).

PCR Amplification.

For PCR using the oligonucleotide of SEQ ID NO:3,
30 oligonucleotide primers with the sequences:
primer 1 5'- UUUUUUUUUUUUUUUUUU -3' (SEQ ID NO:4)
primer 2 5'-Biotin-CCTCCCCTATCTTCTCCT (SEQ ID NO:5)
can be used. Primers were synthesized with an automatic synthesizer (Applied Biosystem, Inc. (Foster City, CA)
35 Model 480A) and purified by electrophoresis in 10% PAGE containing 7 M urea with TBE buffer (0.045M Tris-borate,

0.001 EDTA, pH8.3). Oligonucleotides were recovered from the gel by electroelution.

PCR is conducted, briefly as follows: 20-50 pmol of each primer was added to the reaction mixture. PCR consisted of 30 cycles as follows: 94°C for 45 sec, 65°C for 20 sec, and 72°C for 1 min. This protocol was designed especially for this particular sequence. However, other PCR protocols can yield satisfactory results for the present methods (see, for example Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

LCR Amplification.

For LCR, oligonucleotides with the sequences:
oligo 1 5'-Biotin-CCTCCCCTATCTTCTCCTCCCA (SEQ ID NO:6)
oligo 2 5'-ACCCCAAAAAAAAAAAAAAAAAA (SEQ ID NO:7)
oligo 3 5'-UUUUUUUUUUUUUUUUUUUGGGGT (SEQ ID NO:8)
oligo 4 5'-TGGGGACCAGAAGATAGGGGAGG (SEQ ID NO:9)
can be used. These oligonucleotides can be generated as described above.

Briefly, an example of an LCR protocol is as provided in Barany, F. "Genetic disease detection and DNA amplification using cloned thermostable ligase" (Proc. Natl. Acad. Sci. USA 88:189-193, 1991): The reaction was incubated with thermostable DNA ligase at 94°C for 1 min followed by 65°C for 4 min, and this cycle was repeated 20 times. Other protocols for LCR are well known in the art and can be applied to the present methods with routine modifications.

Following the appropriate number of cycles of amplification, large amounts of a product will be obtained. The PCR or LCR product will have the following structure:

5'-Biotin-CCTCCCCTTTCTTCTCCTCCCCAACCCCAAAAAAAAAAAAAAAAAA-
3'

3'-GGAGGGGAAAGAAGAGGAGGGGTGGGGUUUUUUUUUUUUUUUUUU-
5'

5 (SEQ ID NO:10).

The sensitivity of NATIA is based on the efficiency of amplification using PCR and LCR to amplify nucleic acids. The inconvenience of special thermal conditions associated with both PCR and LCR amplification steps complicates the application of the present method to routine diagnostics. Recently, new methods of isothermal amplification have been described (see, for example, Wolcott, M. J. "Advances in nucleic acid-based detection methods", Clinical Microbiology Reviews, 5(4):370-386, 1992).

Isothermal amplification.

In 3SR amplification reverse transcriptase and RNA polymerase work together to amplify a DNA or RNA template without thermal cyler. The reaction is carried out at constant temperature 37°C. Guatelli et al. "Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication" Pros. Natl. Acad. Sci. USA 87:1874-1878, 1990.

Alternatively, amplification with Q-beta replicase is based on the use of the extraordinary efficiency of the Q-beta polymerase to replicate in vitro Q-beta phage DNA. Sequences specific to the oligonucleotide linked to the immunoreactive component of the NATIA has to be inserted in the genome of the Q-beta phage. By this sequence the phage DNA will be bound to the oligonucleotide moiety adsorbed to a solid-phase. After thorough washing of unbound phage DNA, this DNA may be replicated by Q-beta polymerase (see, for example, Wolcott, M.J. "DNA-based

rapid methods for the detection of foodborne pathogens" J. Food Prot. 54:387-401, 1991).

Strand displacement amplification based on the
5 creation of a nick at specific place in a double-stranded
DNA by a restriction endonuclease and use of the nicked
DNA as a template for a DNA polymerase possessing DNA
strand displacement activity. For initiation of this
amplification reaction, a specific primer is annealed to
10 the oligonucleotide-linked polypeptide (ligand or
antigen). See, for example, Walker et al. "Isothermal in
vitro amplification of DNA by restriction enzyme DNA
polymerase system" Proc. Natl. Acad. Sci. USA 89:392-396,
1992. Other methods of isothermal amplification will also
15 be usable in the claimed methods.

Application of isothermal amplification of nucleic
acids to NATIA would significantly simplify the entire
procedure, allowing the entire reaction to be carried out
20 in one microtiter well without the requirement of
transferring the components from wells into tubes.

Detection of the amplified product.

When treated with uracil DNA glycosylase to remove
25 uracils from the nucleic acid, the product is converted
into a partially double-stranded DNA fragment with an
oligo(dA) 3'-protrusion:

5'-Biotin-CCTCCCCTTTCTTCTCCTCCCCAACCCCAAAAAAAAAAAAAAAAAA-
3'

30 3'-GGAGGGGAAAGAAGAGGAGGGGTTGGGG
(SEQ ID NO:11).

This final product is captured with oligo(dT)
attached to a solid-phase (e.g. microtiter wells). The
35 method for attaching the oligo(dT) to the solid support is
similar to the method used for linking an oligonucleotide
to a synthetic peptide. Special CovaLink NH (Nunc) plates

are used to attach an oligonucleotide to the surface of microtiter plates. Rasmussen et al. "Covalent immobilization of DNA onto polystyrene microwells: the molecules are only bound to the 5'-end" Analytical
5 Biochemistry 198:138-142, 1991.

In another method, briefly, plates are coated with biotinylated bovine serum albumin 9B-BSA. Then, streptavidin is added to the wells to be bound to the
10 biotin linked to BSA. INAA is used to capture biotinylated oligo(dT). Finally, a complex B-BSA-streptavidin-oligo(dT) is attached to the solid-phase and used for the solid-phase detection of reaction amplification products as described elsewhere herein.

15

The captured product is detected by adding the reporter enzyme, horseradish peroxidase, conjugated to streptavidin to the captured product. Briefly, one primer for PCR has an oligo(dU)-tail. The second oligonucleotide
20 is labeled with biotin (or e.g., digoxigenin). A PCR product obtained with the use of these primers will contain on one end biotin (or another moiety for detection) and oligo(dU)-oligo(dA)-tail. After treatment with Uracil DNA glycosylase (UDG), oligo(dU) will be
25 removed allowing exposure of the oligo(dA) in single-stranded form for interaction with oligo(dT) linked to a solid-phase (solid support).

Transcription detection of the reacted oligonucleotide.

30 In addition to amplification by PCR, LCR or isothermal amplification, transcription can be used to detect the immunoreaction of the antigen and antibody in NATIA. For example, the oligonucleotide component of the INAA composition can be designed to include a promoter of
35 the T7 phage ligated to a short specifically designed sequence that can be transcribed using T7 RNA polymerase. The promoter of the T7 phage or SP6 phage is a very short

sequence of about 20 - 30 nucleotides long. This sequence can be designed in the synthetic oligonucleotide to be recognized with T7 or SP6 RNA polymerase. When recognized by RNA polymerase this promoter will induce synthesis of
5 RNA transcript from the oligonucleotide. This transcript may be detected by different methods including gel electrophoresis, or solid-phase detection as described above but with one modification: there is no need to treat the transcript with UDG. This transcript may be designed
10 to contain oligo(A) sequence for the capture with oligo(dT) on solid-phase. Transcript can be labeled for the detection with radioactive nucleotides. Krupp, G. "RNA synthesis: strategy for the use of bacteriophage RNA polymerase" Gene 72:75-89, 1988.

15

Detection of Anti-HCV IgM

Preparation of specimen.

Anti-HCV (IgM) antibodies are captured by anti-mu
20 antibodies attached to the surface of the microtiter wells. Briefly, anti-mu antibodies are diluted in PBS 1:1000 and applied to the microtiter plates wells, incubated overnight at room temperature. After washing the wells to remove unbound anti-mu, specimens are added
25 to the wells at different dilutions. The serum specimen may be diluted 1000 times greater than conventional methods of IgM detection. This extreme dilution should decrease the "noise signal" without any deletion effect on sensitivity of the NATIA.

30

Immunoreaction of specimen and INAA.

The conditions of the immunoreaction are as described above. Briefly, the captured IgM antibody is incubated with an HCV-specific antigen conjugated to a specifically
35 designed oligonucleotide to form the INAA as described above. To additionally diminish the "noise", the INAA also can be added to the reaction in a very diluted form.

The INAA is allowed to react with any captured anti-HCV antibody and the unreacted INAA removed as described above.

5 Detection of the immunoreaction

The oligonucleotide tag is then amplified and the product detected or otherwise detected as described above.

NATIA Using Biotinylated Peptide

10

Preparation of INAA.

A synthetic peptide from the NS4 protein of the hepatitis C virus with structure IIPDREVLVREFDEMEECSQ (SEQ ID NO:12) is biotinylated using a commercially available
15 kit (Pierce, Rockford, Illinois). This peptide was selected because biotinylation does not affect the immunoreactivity of the peptide. A biotinylated oligonucleotide with structure 5'-biotin-
TTTTTTTTTTCAGAAAGCGTCTAGCCATGGCGTTCACCTGTGGTACTGCCTGATA
20 GGG-3' (SEQ ID NO:13) was prepared as described above. This oligonucleotide is composed of sequences of two PCR primers, which are known to work well in PCR, separated by a short additional sequence. The shorter the region to be amplified the better the efficiency of amplification
25 obtained. The biotinylated oligonucleotide is preincubated with streptavidin (Pierce, Rockford, Illinois). Then, this complex is incubated with the biotinylated peptide resulting in formation of an oligonucleotide-peptide complex linked by biotin-
30 streptavidin binding. This INAA complex is then used in place of chemically prepared oligonucleotide-peptide conjugates mentioned above.

Two approaches, antibody capture and direct
35 adsorption, are used to demonstrate the feasibility of the NATIA.

Antibody capture and Immunoreaction.

Anti-human antibodies (anti-IgM, -IgG etc.) are adsorbed on the surface of microcentrifuge tubes and used to capture antibodies from human sera specimens as described above. Then, the tubes are incubated with the peptide-streptavidin -oligonucleotide complex as described above.

Amplification of reacted oligonucleotide.

- After washing, PCR is performed, as described above, using two primers
5'-Digoxigenin-CCCTATCAGGCAGTACCACAA-3' (SEQ ID NO:14) and
5'-UUUUUUUUUUUCAGAAAGCGTCTAGCCATGGCGTT-3' (SEQ ID NO:15).
- The primer labeled with digoxigenin could be labeled with another moiety, for example, biotin. The other primer contains a poly(U)-tail.

Detection of amplified product.

- The resulting PCR product is treated with Uracil-DNA glycosylase as described above to produce an oligo(A)-protrusion on one side and digoxigenin (or biotin) on the other and can be detected by PSSPD (primer specific solid-phase detection). Briefly, different aliquots of the amplification reaction mixture, usually 4 μ l, were treated with 1 unit of UDG (Gibco BRL, Great Island, New York) and 1 μ l of MRP-SA (Gibco BRL, Great Island, New York) in 1xUDG buffer containing 30 mM tris-HCl, pH8.0, 50 mM KCl, 5 mM magnesium chloride for 10 min at 37 C. Aliquots of 1-5 μ l of the reaction mixture were transferred into microtiter wells covered with oligo(dT)20 (Cambridge Biotech, Bethesda, MA) and incubated in 100 μ l of the binding buffer (10 mM tris-HCl, pH7.5, 0.5 M sodium chloride, 0.5% sodium dodecyl sulphate) for 10-30 min at room temperature. Plates were thoroughly washed 5 times with binding buffer. PCR products captured to the surface

of the wells were detected by the addition of the appropriate substrate for HRP.

Direct adsorption and Immunoreaction.

5 Sera diluted with phosphate buffered saline are directly adsorbed on the surface of the microcentrifuge tubes. The adsorption step is as described above and as routinely practiced in the art. The immunoreaction, amplification and detection steps are as described above.

10

In both cases positive results were obtained.

Detection of Hepatitis C Virions

15 Another application of the NATIA is the direct detection of HC virions (undetectable by existing immunodiagnostic methods) in sera specimens.

Specimen preparation.

20 Serum preparation to detect virions is similar to the specimen preparation for detection of antigens. The virion can be captured by HCV specific antibodies preadsorbed on the solid-phase as was described above for capture of IgM. After this step, an HCV specific antibody
25 linked with an oligonucleotide by any of described above approaches may be added.

Preparation of INAA.

30 In this example, an antibody reactive with an HCV envelope protein is covalently linked (conjugated) to the nucleic acid tag (oligonucleotide). Conjugation of the antibody to the oligonucleotide is accomplished in the same manner as described above for the covalent linking of peptides and oligonucleotides.

35

Immunoreaction and detection of reacted INAA.

The conditions for the immunoreaction, amplification and detection of amplified product or transcript are as described above.

5

The above-described methods can be applied to detect other viruses, micro-organisms or disease markers, simply by utilizing an antigenic synthetic peptide, antigen, antibody or ligand specifically reactive with the
10 substance to be detected.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into
15 this application in order to more fully describe the state of the art to which this invention pertains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: The Government of the United States of America, as represented by The Secretary,
Department of Health and Human Services
- (B) STREET: 6011 Executive Blvd., Suite 325
- (C) CITY: Rockville
- (D) STATE: Maryland
- (E) COUNTRY: United States of America
- (F) POSTAL CODE (ZIP): 20852
- (G) TELEPHONE: 301/496-7056
- (H) TELEFAX: 301/402-0220
- (I) TELEX: none

(ii) TITLE OF INVENTION: "NUCLEIC ACID TAGGED IMMUNOASSAY"

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: NEEDLE & ROSENBERG, P.C.
- (B) STREET: Suite 1200, 127 Peachtree Street
- (C) CITY: Atlanta
- (D) STATE: Georgia
- (E) COUNTRY: USA
- (F) ZIP: 30303

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: not yet assigned

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/061,694
- (B) FILING DATE: 13-MAY-1993

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Asn Pro Pro Asp His Ser Ala Pro Leu Gly Val Thr Arg Pro Ser
1 5 10 15

Ala

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Phe Ala Ser Arg Gly Asn His Val Ser Pro Thr His Tyr Val Pro
1 5 10 15

Glu Ser Asp Ala
20

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCCCCTAT CTTCTCCTCC CCAACCCCAA AAAAAAAAAA AAAAAA

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

UUUUUUUUUU UUUUUUUU

18

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCTCCCCTAT CTTCTCCT

18

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCTCCCCTAT CTTCTCCTCC CCA

23

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCCCAAAA AAAAAAAAAA AAA

23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

UUUUUUUUUU UUUUUUUUGG GGT

23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGGGGACCAG AAGATAGGGG AGG

23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: double stranded oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCTCCCCTTT CTTCTCCTCC CCAACCCCAA AAAAAAAAAA AAAAAA

46

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ile Ile Pro Asp Arg Glu Val Leu Tyr Arg Glu Phe Asp Glu Met Glu
 1 5 10 15
 Glu Cys Ser Gln
 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 58 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTTTTTTTT CAGAAAGCGT CTAGCCATGG CGTTCATTG TGGTACTGCC TGATAGGG 58

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCTATCAGG CAGTACCACA A 21

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 37 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

UUUUUUUUUU UUUCAGAAAG CGTCTAGCCA TGGCGTT 37

What is claimed is:

1. A method for detecting the presence of an antigen in a specimen from a subject comprising the steps of:
 - (a) binding antigens in a specimen from the subject to a solid support;
 - (b) contacting the bound antigens with a specifically immunoreactive ligand, wherein the ligand is linked to an oligonucleotide, having a known sequence, under conditions that permit reaction of the oligonucleotide-linked ligand with the bound antigen;
 - (c) removing unreacted oligonucleotide-linked ligand; and
 - (d) detecting the presence of the oligonucleotide, the presence of the oligonucleotide indicating the presence of the antigen in the subject.
2. The method of claim 1, wherein the oligonucleotide and the ligand are linked by biotin-streptavidin-biotin.
3. The method of claim 1, wherein the oligonucleotide is amplified prior to detection.
4. The method of claim 3, wherein the amplification is by polymerase chain reaction.
5. The method of claim 3, wherein the amplification is by ligase chain reaction.
6. A method for detecting the presence of an antibody in a specimen from a subject comprising the steps of:
 - (a) binding antibodies in a specimen from the subject to a solid support;
 - (b) contacting the bound antibodies with a specifically immunoreactive antigen, wherein the antigen is linked to an oligonucleotide, having a known sequence, under conditions that permit reaction of the

oligonucleotide-linked antigen with the bound antibody from the subject;

(c) removing unreacted oligonucleotide-linked antigen; and

(d) detecting the presence of the oligonucleotide, the presence of the oligonucleotide indicating the presence of the antibody in the subject.

7. The method of claim 6, wherein the oligonucleotide and the antigen are linked covalently by chemical reaction.

8. The method of claim 6, wherein the oligonucleotide is amplified prior to detection.

9. The method of claim 8, wherein the amplification is by polymerase chain reaction.

10. The method of claim 8, wherein the amplification is by ligase chain reaction.

11. A composition comprising an oligonucleotide covalently linked to the polypeptide consisting essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:1.

12. A composition comprising an oligonucleotide covalently linked to the polypeptide consisting essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:2.

13. An isolated nucleic acid comprising essentially the nucleotide sequence defined in the Sequence Listing by SEQ ID NO:3.

14. A composition comprising an immunoreactive polypeptide covalently linked to the nucleic acid of claim 7.
15. The composition of claim 14, wherein the polypeptide consists essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:1.
16. The composition of claim 14, wherein the polypeptide consists essentially of the amino acid sequence defined in the Sequence Listing by SEQ ID NO:2.
17. A method for detecting current or previous hepatitis C virus infection in a subject, comprising the steps of:
 - (a) binding antibodies in a specimen from the subject to a solid support;
 - (b) contacting the bound antibodies with the composition of claim 12 under conditions that permit the reaction of the composition of claim 12 with an antibody from the subject;
 - (c) removing the unreacted composition of claim 12,
 - (d) detecting the presence of the oligonucleotide of the composition, the presence of the oligonucleotide indicating the reaction of the polypeptide of claim 12 and an antibody from the subject, the reaction indicating current or previous hepatitis C virus infection.
18. The method of claim 11, wherein the oligonucleotide is amplified prior to detection.
19. The method of claim 12, wherein the amplification is by polymerase chain reaction.
20. The method of claim 17, wherein the amplification is by ligase chain reaction.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12Q1/68 G01N33/576 //C07H21/04, C07K7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCIENCE. vol. 260 , 30 April 1993 , LANCASTER, PA US pages 698 - 99 RUZICKA V. ET AL 'Immuno-PCR with a commercially available avidin system' cited in the application see the whole document ---	1-10
X	SCIENCE. vol. 258 , 2 October 1992 , LANCASTER, PA US pages 120 - 122 SANO, T. ET AL 'Immuno-PCR: Very sensitive antigen detection by means of specific antibody-DNA conjugates' cited in the application see the whole document --- -/--	1-10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

6 September 1994

Date of mailing of the international search report

26 -09- 1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

Osborne, H

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 9135, Derwent Publications Ltd., London, GB; AN 91-256837 & JP,A,3 167 474 (HITACHI KK) 19 July 1991 see abstract ---	1-10
X	WO,A,91 17442 (CHIRON CORP.) 14 November 1991 A see the whole document	1,3,6-8 4,5,9, 10,17-19
P,X	EP,A,0 544 212 (NISSHIN FLOUR MILLING CO. LTD.) 2 June 1993 see the whole document ---	1-10
P,X	WO,A,93 15229 (E.I. DU PONT DE NEMOURS AND CO.) 5 August 1993 see the whole document especially pages 24-29 ---	1-10
A	WO,A,93 06488 (GENELABS TECHNOLOGIES INC.) 1 April 1993 see page 122, line 1; claims 1-5,26 ---	12,14, 16,17
A	WO,A,92 00989 (IMPERIAL CHEMICAL INDUSTRIES PLC) 23 January 1992 -----	

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9117442	14-11-91	AU-A- 7791291 EP-A- 0528870 HU-A- 64623	27-11-91 03-03-93 28-01-94
EP-A-0544212	02-06-93	JP-A- 5149949	15-06-93
WO-A-9315229	05-08-93	AU-B- 3618593	01-09-93
WO-A-9306488	01-04-93	AU-A- 2683792	27-04-93
WO-A-9200989	23-01-92	NONE	